

(12) **UK Patent Application** (19) **GB** (11) **2 233 977** (13) **A**

(43) Date of A publication 23.01.1991

(21) Application No 8900107.7

(22) Date of filing 04.01.1989

(71) Applicants

Michael Kehoe
14 Springhouse Lane, Ebchester, County Durham,
DH8 0QF, United Kingdom

Michael Pinkney
6 Ilfracombe Gardens, Whitley Bay, Tyne and Wear,
NE26 3SL, United Kingdom

(72) Inventors

Michael Kehoe
Michael Pinkney

(74) Agent and/or Address for Service

Mewburn Ellis
2 Cursitor Street, London, EC4A 1BQ, United Kingdom

(51) INT CL⁵

C12N 15/31, C07K 13/00 // (C12N 15/31 C12R 1:19)

(52) UK CL (Edition K)

C3H HB7M H650 H674
C6Y Y125 Y501 Y503
U1S S1337 S2419

(56) Documents cited

GB 2226563 A
Infect. Immun 1989, 57(8),
2553-2558
Infect. Immun 1987, 55 (12),
3228-3232

(58) Field of search

UK CL (Edition K) C3H HB7M
INT CL⁵ C07K 13/00 15/00, C12N 15/31
Online databases: WPI, DIALOG/BIOTECH

(54) **Cysteine-free streptolysin O**

(57) A derivative of the thiol-activated toxin streptolysin O, which contains no cysteine amino acids but retains cytolytic activity which can be neutralised by anti-SLO antibodies in a sample.

Figure -

A NATIVE SLO SEQUENCE

SLO.DNA

TTATTAGCAAGCTTGCCATTTTATTTTAAACCGTCAAAGCATACTAGCTAATATAACCAAA 60

BCGTTAAAGATGCGCATTATTAGAGAGGCTATGGCACATTACAAATTAGGAGAATTTGC 120

TCACTATCAAGATACTATGCTTGATATGGTCGAAAGAACAATAGAAACATTTTAGAATGA 180

TAAAAAGGTATGAAGGACATGTCTAATAAAAAACATTTAAAAAATACAGTCGCGTCGCT 240
M S N K K T F K K Y S R V A

GGGCTACTGACGGCAGCTCTTATCATTGGTAACCTTGTTACTGCTAATGCTGAATCGAAC 300
G L L T A A L I I G N L V T A N A E S N

AAACAAAACACTGCTAGTACAGAAACCACAAACGACAAATGAGCAACCAAGCCAGAAAGT 360
K Q N T A S T E T T T T N E Q P K F E S

AGTGAGCTAACTACTGAAAAAGCAGGTCAGAAAACGGATGATATGCTTAACTCTAACGAT 420
S E L T T E K A G Q K T D D M L N S N D

ATGATTAAGCTTGCTCCCAAGAAATGCCACTAGAATCTGCAGAAAAAGAAAGAAAAAAG 480
M I K L A P K E M P L E S A E K E E K K

TCAGAAAGACAAAAAAGAGCGAAGAAGATCACACTGAAGAAATCAATGACAAGATTTAT 540
S E D K K K S E E D H T E E I N D K I Y

TCACTAAATTATAATGAGCTTGAAGTACTTGCTAAAAATGGTGAAACCAATTGAAAATTTT 600
S L N Y N E L E V L A K N G E T I E N F

GTTCTAAAGGAGGCGTTAAGAAAGCTGATAAATTTATTGTCATTGAAAGAAAGAAAAAA 660
V P K E G V K K A D K F I V I E R K K K

AATATCAACACTACACCAAGTCGATATTTCCATCATTGACTCTGTCACTGATAGGACCTAT 720
N I N T T P V D I S I I D S V T D R T Y

CCAGCAGCCCTTCAGCTGGCTAATAAAGGTTTTACCGAAAACAAACCAGACGCGGTAGTC 780
P A A L Q L A N K G F T E N K P D A V V

ACCAAGCGAAACCCACAAAAAATCCATATTGATTTACCAGGTATGGGAGACAAAGCAACG 840
T K R N P Q K I H I D L P G M G D K A T

GTTGAGGTCAATGACCCTACCTATGCCAATGTTTCAACAGCTATTGATAATCTTGTTAAC 900
V E V N D P T Y A N V S T A I D N L V N

960
 CAGTGGCATGATAATTATTCTGGTGGTAATACGCTTCCTGCCAGAACACAATATACTGAA
 L W H D N Y S G G N T L P A R T Q Y T E

1020
 TCAATGGTATATTCTAAGTCACAGATTGAAGCAGCTCTAAATGTTAATAGCAAAATCTTA
 S M V Y S K S Q I E A A L N V N S K I L

1080
 GATGGTACTTTAGGCATTGATTTCAAGTCGATTTCAAAAGGTGAAAAGAAGGTGATGATT
 D G T L G I D F K S I S K G E K K V M I

1140
 GCAGCATACAAGCAAATTTTACACCGTATCAGCAAACCTTCCTAATAATCCTGCCGGAT
 A A Y K Q I F Y T V S A N L F N N P A D

1200
 GTGTTTGATAAATCAGTGACCTTTAAAGAGTTGCAACGAAAAGGTGTCAGCAATGAAGCT
 V F D K S V T F K E L Q R K G V S N E A

1260
 CCGCCACTCTTTGTGAGTAACGTAGCCTATGGTCGAACTGTTTTTGTCAAACCTAGAAACA
 P P L F V S N V A Y G R T V F V K L E T

1320
 AGTTCTAAAAGTAATGATGTTGAAGCGGCCTTTAGTGCACTCTAAAAGGAACAGATGTT
 S S K S N D V E A A F S A A L K G T D V

1380
 AAAACTAATGGAAAATACTCTGATATCTTAGAAAATAGCTCATTACAGCTGTCGTTTTTA
 K T N G K Y S D I L E N S S F T A V V L

1440
 GGAGGAGATGCTGCAGAGCACAATAAGGTAGTCACAAAAGACTTTGATGTTATTAGAAAC
 G G D A A E H N K V V T K D F D V I R N

1500
 GTTATCAAAGACAATGCTACCTTCAGTAGAAAAAACCAGCTTATCCTATTTTCATACACC
 V I K D N A T F S R K N P A Y F I S Y T

1560
 AGTGTTCCTTAAAAATAATAAAATTGCGGGTGTCAATAACAGAACTGAATACGTTGAA
 S V F L K N N K I A G V N N R T E Y V E

1620
 ACAACATCTACCGAGTACACTAGTGGAATAAATTAACCTGTCTCATCAAGGCGCGTATGTT
 T T S T E Y T S G K I N L S H Q G A Y V

1680
 GCTCAATATGAAATCCTTTGGGATGAAATCAATTATGATGACAAAGGAAAAGAAGTGATT
 A Q Y E I L W D E I N Y D D K G K E V I

1740
 ACAAACGACGTTGGGATAACAACTGGTATAGTAAGACATCACCATTTAGCACAGTTATC
 T K R R W D N N W Y S K T S P F S T V I

1800
 CCACTAGGAGCTAATTCACGAAATATACGTATCATGGCTAGAGAGGCCACCGGCTTAGCT
 P L G A N S R N I R I M A R E A T G L A

1860
TGATGGTGGCGAAAAGTGATCGACGAAAGAGATGTGAAACTGTCTAAAGAAATCAAT
W E W W R K V I D E R D V K L S K E I N

1920
GTCAACATCTCAGGATCAACCCTGAGCCCATATGGTTTCGATTACTTATAAGTAGGACTGG
V N I S G S T L S P Y G S I T Y K

TTCAAGAGGTTC

CYTOLYTIC STREPTOLYSIN O MUTANTS AND USES

This invention relates to cytolytic streptolysin O (SLO) mutants and their uses in diagnostic anti-streptolysin O antibody tests (ASO tests). In particular the present invention relates to the identification, construction and production of cytolytic SLO derivatives that do not contain any cysteine (Cys) amino acids. This invention also relates to the use of these SLO derivatives in diagnostic tests based on specific binding properties, such as binding between antigen and antibody, and the inhibition of the cytolytic activities of SLO and cytolytic SLO derivatives by anti-streptolysin O antibodies in human serum.

SLO is a toxic cytolytic protein produced by *Streptococcus pyogenes* (*S.pyogenes*) which causes a number of human diseases. During infection, the gene encoding SLO is expressed and SLO is secreted by *S.pyogenes*. The SLO secreted by *S.pyogenes* is sensitive to oxidation and the cytolytic activity is greatly reduced after a period of exposure to air. This lost activity is restored by the addition of reducing agents such as dithiothreitol (DDT). The sensitivity of SLO to oxidation is associated with the presence of a Cys amino acid in the native protein. All prior art indicates that this Cys amino acid is an essential amino acid for the cytolytic activity of SLO and this Cys amino acid is often referred to as the "essential Cys".

The infected human host produces anti-SLO antibodies to antigenic sites on the SLO molecule. Thus diagnostic tests detecting these anti-SLO antibodies in human serum, can indicate (past or present) infection by *S.pyogenes*.

The immunodiagnostic assays presently being used for detection of anti-SLO antibodies in human serum utilise active SLO protein prepared from cultures of *S.pyogenes*. These assays generally comprise the following steps:

- (a) Take serum sample from patient.
- (b) Make serial dilutions of serum sample in a suitable buffer.
- (c) For each test include a control containing buffer but no serum.
- (d) Add a standard quantity of active SLO to each dilution of serum and to the control.
- (e) Incubate the mixtures for a standard time and at a standard temperature, to allow any anti-SLO antibodies in the mixtures to combine with, and neutralise the added SLO.
- (f) Add a standard quantity of red blood cells to each mixture.
- (g) Incubate the mixtures for a standard time and at a standard temperature to allow any active (non-neutralised) SLO to lyse the added cells.
- (h) Determine the highest dilution of serum that has neutralised the added SLO, that is which corresponds to the dilution producing less than 50% lysis of the added red blood cells.

Thus, where the serum sample contains high levels of anti-SLO antibody, there will be neutralisation of the active SLO and therefore no lysis of the red blood cells. Conversely, where the serum sample does not contain antibodies to SLO, there will be no neutralisation and therefore there will be extensive lysis of the

red blood cells.

There are a number of problems with preparing active SLO from *S.pyogenes* cultures for use in these assays. These problems are caused by the sensitivity of SLO to degradation by proteases that are also secreted by *S.pyogenes* and by the sensitivity of SLO to oxidation due to the presence of a Cys amino acid in the molecule. The gene encoding SLO has been cloned and expressed in *Escherichia coli* (*E.coli*) and the complete nucleotide sequence of the cloned SLO gene has been determined and the amino acid sequence of SLO has been deduced from the nucleotide sequence of the gene. These data have been published (Kehoe and Timmis, 1984, Infection and Immunity 43:804-810 and Kehoe *et al* 1987, Infection and immunity 55:3228-3232). These data showed that the SLO protein contains only one Cys amino acid. The native SLO expressed by the cloned gene in *E.coli* could be used in the current immunodiagnostic assays. However the cloned gene product also contains the Cys amino acid. Prior art indicates that deletion of this single Cys amino acid or the exchange of this Cys amino acid for any other natural amino acid would irreversibly inactivate the cytolytic activity of SLO.

The problem addressed by the present application was that of producing a cytolytic derivative of SLO did not contain a Cys amino acid and that was not sensitive to oxidation. It was not at all clear that this could be done.

In one aspect the present invention provides data that shows that Cys is not an essential amino acid for the cytolytic activity of SLO.

In another aspect the present invention provides a DNA sequence encoding a cytolytic SLO derivative that does not contain a Cys amino acid.

In another aspect the present invention provides a cytolytic SLO derivative that does not contain a Cys amino acid.

In another aspect the present invention provides a process which comprises producing cytolytic SLO derivatives that do not contain a Cys amino acid by culturing a transformed cell so as to express the protein and recovering the protein therefrom.

The present invention also provides methods and diagnostic kits for detecting the presence or absence of antibodies to SLO in clinical samples and for quantifying the levels of such antibodies in these samples. A typical diagnostic method may comprise replacing native SLO in the current ASO assays (described above) with the cytolytic SLO derivatives that do not contain a Cys amino acid.

In order that the present invention may be more readily understood, embodiments will now be described with reference to Figure 1 which shows how the genes encoding cytolytic SLO derivatives (referred to as "MUTANT" in Fig. 1) were constructed and with reference to

Figure 2 which shows the complete nucleotide sequences of the cloned SLO gene and a mutant gene encoding a cytolytic SLO derivative that does not contain a Cys amino acid.

Figure 1 outlined the steps involved in constructing mutant SLO genes encoding SLO derivatives that do not contain a Cys amino acid. Plasmid pMK206 DNA was cleaved with the restriction enzymes SnaBI (S) and BamHI (B) and the two resulting DNA fragments were separated by electrophoresis on agarose gels and purified (highlighted by boxes). The smaller of these two purified DNA fragments was then cleaved with the restriction enzyme HpaII (Hp) and the two resulting fragments were separated. The larger Hp-B fragment was purified and the smaller S-Hp fragment was discarded. A double stranded oligonucleotide was prepared by DNA synthesis *in vitro* and hybridisation, such that it replaced the discarded segment of the SLO gene with a new sequence that did not contain a Cys amino acid codon, but that replaced this Cys codon with a codon for an alanine or some other amino acid. The purified large B-S fragment from the first restriction enzyme cleavage, the purified Hp-B fragment from the second restriction enzyme cleavage and the synthetic double stranded oligonucleotides were ligated together to produce new plasmids. These new recombinant plasmids were transformed into an *E.coli* host and progeny expressing cytolytic SLO derivatives that do not contain a Cys amino acid were identified. The sequence of one such derivative, where the Cys codon is replaced by a codon for the amino acid alanine is described in Figure 2. A procedure for producing the cytolytic SLO derivatives from the transformed *E.coli* cells was developed such that protein that could be used in immunodiagnostic tests could be provided.

APPLICATION NO: 8900107.7
FILING DATE: 04.01.1989
M. KEHOE and M. PINKNEY.

CLAIMS

1. A cytolytic derivative of the thiol-activated protein streptolysin O (SLO) comprising amino acid sequences with no cysteine (Cys) amino acid.
2. An SLO derivative according to claim 1 wherein SLO has been subjected to derivatisation at the Cys amino acid.
3. An SLO derivative according to claim 2 wherein the derivatisation involves altering the amino acid sequence by means amino acid substitution, insertion or deletion achieved by genetic manipulation of an SLO gene or any other means.
4. An SLO derivative according to claim 1 wherein the cytolytic activity is resistant to inactivation by oxidation or chemical modification of a thiol group.
5. A process which comprises the production of an SLO derivative of claim 3 by expression of the protein in a recombinant host cell from DNA encoding the derivative.
6. A diagnostic kit for detecting the presence or absence of antibodies to SLO in a clinical sample, which comprises an SLO derivative of claim 1 together with ancillary components for measuring cytolytic activity.
7. A method for detecting the presence or absence of antibodies to SLO in a clinical sample, which method comprises contacting dilutions of the sample with an SLO derivative of claim 1 and measuring the cytolytic activity remaining after contact between the sample dilutions and the SLO derivative of claim 1.